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PRINCIPAL INVESTIGATOR: Victoria Sung

CONTRACTING ORGANIZATION: Georgetown University
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Bone Sialoprotein (BSP), a secreted glycoprotein normally found in bone matrix, is implicated in the formation of mammary gland microcalcifications and localized to approximately 80% of primary human breast cancers.

This project utilized cDNA transfection methodology to examine the role of BSP in human breast cancer invasiveness and bone metastasis.

In addition, the project characterized a new human breast cancer cell lines isolated from a metastatic lesion in the femur.

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Table of Contents

I. Standard Form 298

II. Foreword

III. Introduction

IV. Body

A. Experimental Methods, Assumptions, Procedures
B. Results and Discussion
1. Expression of bone sialoprotein in human breast cancer
a. Localization of BSP to primary breast tumors and their corresponding bone metastases
b. BSP is not expressed by human breast cancer cell lines *in vitro* or *in vivo*
c. Expression of BSP in the MDA-MB-231BAG HBC cell line
d. BSP-expression in MDA-MB-231BAG results in increased migration and diminished adhesion *in vitro*
2. The LCC15-MB human breast cancer cell line expresses osteopontin and exhibits an invasive and metastatic phenotype
a. LCC-15 cells display an invasive phenotype *in vitro*
b. Attachment profile of LCC15-MB cells
c. Osteopontin expression by LCC15-MB cells
d. Expression of the bone matrix protein osteopontin in LCC15-MB xenografts

C. Recommendations

V. Conclusion

VI. References

VII. Appendices

ANNUAL REPORT (1997-98) for Army Grant DAMD17-96-1-6134
Principal Investigator: Victoria Sung

Abstract

Bone sialoprotein (BSP), a secreted glycoprotein normally found in bone matrix, is implicated in the formation of mammary microcalcifications and localized to approximately 80% of primary human breast cancers (HBC). The presence of BSP may play a role in the propensity of certain breast cancers to metastasize, particularly to bone. Although it is present in primary breast lesions and their corresponding bone metastases by immunohistochemistry, we did not detect BSP mRNA by Northern or protein by Western analysis in a panel of human breast cancer (HBC) cell lines either *in vitro* or *in vivo*. Therefore, in order to explore possible functions for BSP in breast cancer invasion and metastasis, the full length cDNA for BSP was transfected into the non-BSP-expressing MDA-MB-231 HBC cell line engineered to express Lac-Z (MDA-231-BAG). Resultant clones selected for BSP expression had increased migratory capacity compared to vector-transfected MDA-MB-231BAG cells, consistent with observed *in vitro* responses of these cells to exogenous BSP. BSP transfectants also adhered less well to plastic and to the extracellular matrix protein vitronectin, suggesting perturbations in the $\alpha v\beta 3$ integrin functionality. These results support the concept that the presence of BSP in HBC may contribute to increased invasiveness and possibly bone metastasis. In a separate project, we have characterized the LCC15-MB cell line which was recently derived from a breast carcinoma metastasis resected from the femur of a 29-year-old woman. The expression of traits commonly associated with metastasis as well as recolonization of bone in an *in vivo* nude mouse model suggests that this novel cell line will be also valuable in the study of breast cancer metastasis.

Subject Terms: Breast cancer, bone sialoprotein, bone, metastasis, migration, adhesion

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1. Sung V, Cattell DA, Bueno J, Murray A, Zwiebel JA, Aaron AD, Thompson EW. 1997. Human breast cancer cell metastasis to long bone and soft organs of nude mice: A quantitative assay. Clin Exp. Metast., 15(2): 173-178.

2. Sung V, Stubbs J, Fisher L, Aaron AD, Thompson EW. 1998. Bone sialoprotein supports breast cancer cell proliferation and migration through differential usage of the alpha v beta 5 and alpha v beta 3 integrins. J. Cell. Phys., in press.

3. Sung V, Gilles C, Murray A, Clarke R, Aaron AD, Azumi N, Thompson EW. 1998. The LCC15-MB human breast cancer cell line expresses osteopontin and exhibits an invasive and metastatic phenotype. Exp. Cell Res., in press.

III. Introduction

Although current treatment for primary breast disease is largely effective, very little is known about the process of tumor metastasis, resulting in a high mortality rate in those patients with secondary dissemination [1]. Bone is the second most common organ in which breast cancer metastases develop, being found at autopsy in over 80% of patients dying from carcinoma of the breast [2]. Both the bone environment and intrinsic properties of a tumor cell are thought to play a role in the metastatic process. For example, the bone marrow is highly vascularized, so that it not only has a higher exposure to tumor cells, but also to various systemic hormones, cytokines and growth factors which may promote growth of metastatic cells [3]. The bone itself, is also a reservoir for numerous growth factors, extracellular matrix proteins and cytokines which can be released during remodeling and resorption (rev. in [4]). On the other hand, tumor cells may also contribute to the propensity for metastasis by producing proteolytic enzymes which degrade extracellular (ECM) matrix and basement membrane, exhibiting cell surface recognition molecules which favor arrest in bone, and increasing migratory capability and growth responsiveness to bone-localized growth factors and hormones. Identifying some of these mechanisms involved in the bone metastasis of breast cancer will contribute to a better understanding of this metastatic pathway, and may allow us to eventually prevent the spread of breast malignancies to bone.

Reports in the literature have suggested that the ability of certain breast cancer cells to metastasize to bone may be enhanced by bone sialoprotein (BSP), a small acidic bone matrix protein [5]. Although normally found in the bone as one of the most abundant non-collagenous proteins, and also in human decidua and trophoblast cells, BSP was recently shown to be present in almost 80% of primary mammary carcinomas where it is associated with poor patient survival and the presence of mammary microcalcifications, irregular hydroxyapatite crystals which form in both benign and malignant breast disease, respectively [6-8]. In addition, abnormally high serum BSP levels have been correlated with malignant bone disease [9,10]. Primarily secreted by osteoblasts and osteoclasts, BSP is thought to play a role in mineralization and bone formation by binding to hydroxyapatite [6,11]. The complete nucleic acid sequences of human, rat, mouse and cow, as well

as a partial cDNA sequence for pig BSP have been determined, and each encode a highly modified protein with a conserved integrin-binding RGD (Arg-Gly-Asp) tripeptide of approximately 75-kD. The RGD tripeptide is commonly present in extracellular attachment proteins, and the RGD domain of BSP has been shown to mediate the attachment of osteosarcoma cells and osteoclasts *in vitro* [12-14]. More recently, we have shown that migration, adhesion and proliferation of the HBC cell line, MDA-MB-231, are all dependent on the RGD motif. By using integrin-blocking antibodies and integrin-selected MDA-MB-231 cells, we demonstrated that the $\alpha v\beta 3$ integrin is specifically responsible for BSP-induced cell migration, while the $\alpha v\beta 5$ integrin is more involved in proliferative and adhesive behaviors [15].

Though BSP appears to be present in breast lesions, it is not yet clear how BSP expression in tumor cells might contribute to a selective advantage for metastasis or bone colonization. To study these possibilities, we surveyed several primary breast lesions and their associated bone metastases as well as a number of established HBC cell lines for BSP expression. Although strong immunocytochemical data exist for BSP production by breast carcinoma cells, we were unable to find BSP mRNA or protein in a panel of HBC cell lines in vitro or in vivo as nude mouse xenografts. Therefore, to begin characterizing the relationships between BSP expression and the process of metastasis, we transfected a non-BSP expressing HBC cell line, MDA-MB-231BAG, with the BSP sense transcript. Subsequent *in vitro* experiments designed to measure important components of the metastatic cascade demonstrated an increased inherent migration and decreased adhesion to specific substrates by HBC cells expressing BSP, as compared to the non-expressing parental and vector control cells.

To further our studies involving the bone metastasis of breast cancer, we have isolated the LCC15-MB cell line. These cells were established from a femoral bone metastasis that arose in a twenty-nine year old woman initially diagnosed with an infiltrating ductal mammary adenocarcinoma. The tumor had a relatively high (8%) S-phase fraction and 1/23 positive lymph nodes (LN). Thirty-five months after the initial diagnosis she was treated for acute skeletal metastasis, and stabilized with a hip replacement. At this time, tumor cells were removed from surplus involved bone, inoculated

into cell culture, and developed into the LCC15-MB cell line. We have shown previously that the LCC15-MB cell line expresses vimentin [16], the intermediate filament protein usually restricted to mesenchymally-derived tissue and which has been strongly correlated with basement membrane invasiveness *in vitro* by a variety of tumor cell lines [17,18]. Vimentin positive cells, as a group, are significantly more invasive than their more epithelial counterparts, and expression of vimentin in epithelial tumors is thought to arise by a process resembling the epithelial to mesenchymal transition (EMT). The EMT, known to transiently occur when epithelial cells need to adopt a migratory, and possibly invasive state during embryogenesis, organ development, and wound healing, has been shown to be regulated by both growth factors and extracellular matrix (ECM) components (reviewed in [19]).

The LCC15-MB cells also express the bone matrix protein, osteopontin (OPN), which may be involved in several steps of breast cancer invasion and metastasis [20] such as tumor cell attachment and migration [21]. OPN, like BSP, is an acidic, sulfated glycoprotein which is primarily secreted by osteoblasts and osteoclasts, and thought to take part in bone resorption and possibly bone formation (rev. in [22]). OPN also contains an RGD integrin-binding domain which has been implicated in osteoclast-mediated bone resorption, initiation of cellular signaling pathways and increased adhesion and migration of HBC cells [21,23,24]. Aside from its RGD-mediated activities, OPN also appears to have non-RGD domains which promote cell attachment [25]. Using the LCC15-MB cell line, we have confirmed our previously determined relationship between VIM expression and breast cancer invasion, and further found that OPN expression is shared by LCC15-MB cells and only the most highly metastatic MDA-MB-435 HBC cell line. We conclude that the presence of both VIM and OPN may contribute to the overall invasive phenotype in breast cancer.

IV. Body

A. Experimental Methods, Assumptions and Procedures

Cell culture. The MDA-MB-231 human breast cancer cell line was originally obtained from ATCC, Rockville, MD and transduced with the BAG retroviral vector [26] as described in Brunner *et al.*, 1992 (MDA-MB-231BAG). The cells were grown in Richter's Improved Minimal Essential Medium (DMEM, Biofluids, Rockville, MD) and supplemented with 10% fetal bovine serum. As the BAG vector encodes both neomycin (G418, Gibco, Gaithersburg, MD) resistance and β-galactosidase (β-gal), the transduced cells could be selected and enriched by culture in G418, and confirmed by X-gal staining. The cells used in this study were passaged continuously for 1-3 months, and confirmed routinely to be at least 80% positive by X-gal staining. This proportion of X-gal positivity persisted under G418 selection. The isolation of LCC15-MB was recently described [16], and the LCC15-MB cells used here were between passages 20 and 30. Other established human breast cancer (HBC) cell lines used in comparison (T47D, MCF-7_{ADR}, SKBr3, MDA-MB-453, BT549, MDA-MB-436, MDA-MB-231, MDA-MB-435, Hs578T) were originally obtained from the ATCC (Rockville, MD). MCF-7 cells, originally obtained from Dr. Marvin Rich (Michigan Cancer Foundation), were provided by the Lombardi Cancer Center Cell Culture Shared Resource and the UMR-106-01BSP rat osteosarcoma cell line was obtained from Dr. Larry Fisher (NIDR, NIH, Bethesda, MD). All cell lines were grown in Richter's Improved Minimal Essential Medium (IMEM, Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and maintained at 37°C in 5% CO_2, 95% O_2. Cultures were confirmed as Mycoplasma free by the Lombardi Cancer Center Cell Culture Shared Resource using the Genprobe kit (Gen-Probe, San Diego, CA).

***In vivo* Models.**

Intracardiac Cell Inoculation Into Nude Mice. To establish experimental bone metastases, LCC15-MB cells were inoculated intracardially (*i.c.*) into the left ventricle of two groups of four NCR *nu/nu* nude mice (4-6 week old) anesthetized by inhalation of methoxyflurane (Pittman Moore,

Mundelein, NJ) as previously described [27]. The cells were routinely prepared for inoculation by pelleting and washing twice by centrifugation followed by resuspension at 100,000 cells/100 μl 1X PBS. Cell viability was determined by trypan blue exclusion to be at least 90% viable. Prior to injection, the cell suspension was agitated using a pipet to disperse aggregates. The anterior chest was scrubbed with ethanol and the left ventricle located by using the second intercostal space and sternum as a guide. A 30-gauge needle with a tuberculin syringe was inserted into the heart, and the spontaneous, pulsatile entrance of oxygenated blood into the needle hub indicated proper positioning of the needle. The tumor cells were then injected, and the mice allowed to recover from anesthesia before being returned to their cages. After 4 weeks, the mice were sacrificed by CO_2 asphyxiation, and the metastasis to different organs was scored on the basis of LCC15-MB cell outgrowth per culture. At the time of harvest, bone was sagittally bisected and soft organs were minced. Both were cultured in IMEM supplemented with 10% FBS and antibiotics, and the cells maintained at 37°C in 5% CO_2, 95% O_2. In a second experiment, samples of soft organs and tumor were fixed in 10% formalin, embedded in paraffin, and analyzed by routine histology (H&E stained) for the presence of metastasis.

Subcutaneous Xenografting in Nude Mice. Mice were housed in sterile laminar flow rooms at 25°C and 50% humidity. Subcutaneous (S/c) xenografting was performed as previously described for other HBC cell lines [17,28]. LCC15-MB cells were harvested from near confluence with trypsin, resuspended in PBS at 1 x 10^7 cells/ml, and inoculated bilaterally (5 x 10^6 cells/site) into the mammary fat pad area of 6-8 week old female NCr *nu/nu* nude mice (NCI, Frederick, MD). Mice were monitored daily, and tumor measurements were taken twice weekly for 30 days. At the time of harvest, samples of soft organs and tumor were fixed in formalin, embedded in paraffin, and analyzed by routine histology (hematoxylin and eosin, H&E).

Immunohistochemistry. Archival paraffin blocks of primary breast tumors and corresponding bone metastases were obtained from the tumor bank of the Lombardi Cancer Center Shared Tissue Resource. Paraffin-embedded plugs of each cell line were prepared by harvesting cells at approximately 80% confluence from 100 mm^3 tissue culture plates, washing with 1X PBS and

immediately fixing in a 10% formalin solution. Nude mouse xenografts were fixed similarly and also embedded in paraffin blocks. Sectioning of the paraffin blocks onto *pro-bond*+ (Fisher Scientific, Pittsburgh, PA) slides was performed by the Lombardi Cancer Center Tissue Culture, Tumor Bank, and Cytochemistry & Microscopy Core Facility. Sections were heated to 60°C and submerged in xylene for 10 minutes (twice) to deparaffinize tissue sections. After clearing in 100% and 70% ethanol, tissue was blocked for 30 minutes in 0.3% hydrogen peroxide (Sigma, St. Louis, MO), washed with 1X PBS and treated with 200 μg/ml proteinase K at 37°C, 10 minutes, to expose the antigen. The sections were blocked for 30 minutes with 2% goat serum (Sigma) prior to addition of BSP (LF-83 or LF100; 1:500 dilution) antisera for 2 hours at room temperature. The BSP and OPN antibodies have been shown to cross react with other species including [29]. An HRP-conjugated goat anti-rabbit secondary antibody (Sigma Immunochemicals, St. Louis, MO; 1:200) was then applied for 1 hour and developed using DAB tablets (Sigma). Finally, sections were counterstained with hematoxylin/eosin, dehydrated, mounted onto glass slides and examined using a Zeiss IM-35 microscope.

Northern Analysis. Cytoplasmic RNA was prepared by standard methods using guanidinium isothiocyanate (Gibco BRL, Gaithersburg, MD) from cultured HBC cell lines as well as nude mouse xenografts [30]. Approximately 20 μg of mRNA was loaded onto 1% agarose gel containing 10% formaldehyde and transferred to a nylon membrane (Hybond-N, Amersham Life Sciences, Arlington Heights, IL). A cDNA probe for BSP (B65g) [29,31] was radioactively labeled with ^{32}P using random hexamer priming (Boerhinger Mannheim, West Germany). The GAPDH probe used to normalize loading was from Clonetech Laboratories, Inc. (Palo Alto, CA).

Western analysis. Whole cell lysates were prepared from HBC cell lines using 0.8 ml/100 mm^2 tissue culture dish of standard extraction buffer (RIPA buffer: 1% PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mg/ml PMSF, 1μg/μl aprotinin, 100 μM sodium orthovanadate (all chemicals from Sigma, St. Louis, MO), separated on a 10% SDS-PAGE gel, and transferred to a nitrocellulose filter (Hybond-ECL membrane, Amersham Life Sciences, Arlington Heights, IL). The filter was blocked with 5% nonfat milk and blotted with rabbit

polyclonal antisera directed against BSP (LF100; dilution of 1:500) [29] followed by incubation with a secondary antibody HRP-conjugated goat anti-rabbit IgG (Sigma Immunochemicals, St. Louis, MO; 1:2500 dilution). Signal was developed using the ECL chemiluminescent reagent (Amersham Life Sciences, Arlington Heights, IL).

Cell Migration Assay. Chemomigration experiments were performed in triplicate essentially as previously described [17,18] except that the 48-well Boyden chamber apparatus (Neuroprobe, Cabin John, MD) was used. These assays were performed using filters coated with a thin layer of Type IV collagen (50 μg/ml; a kind gift from Hynda Kleinman, NIDR, NIH, Bethesda, MD) to allow cell adhesion. IMEM supplemented with 0.1% BSA or 10% FBS was used as the chemoattractant in the lower chamber. Cells were harvested with trypsin (Gibco BRL, Gaithersburg, MD), washed twice with serum-free IMEM, resuspended in IMEM-BSA, and added to the upper chamber at a density of 10,000 cells/well. Chambers were incubated in a humidified incubator at 37°C in 5% CO_2 for 18 hours. The cells which had traversed the filter and spread on its lower surface were stained with Diff-Quik (American Scientific Products, McGaw Park, IL), and quantitated by counting. Data presented are an average (with standard error) number of cells per nine representative microscopic fields viewed at a magnification of 40X.

Cell Attachment Assay. Attachment assays were performed essentially as described [28]. Prior to the assay, 50 μl/well of diluted vitronectin (a gift from Dr. Steve Akiyama, NIDR, NIH), fibronectin (Collaborative Biomedical Products, Bedford, MA) and collagen IV (a gift from Dr. Hynda Kleinman, NIDR, NIH) was added to triplicate wells in a 96-well tissue culture plate followed by incubation for 1 hour at 37°C to precoat the wells with substrate. Non-coated wells were submerged in 1X PBS. 50 μl of 3% BSA in PBS was added to each well to block non-specific binding sites and the plate incubated for 30 minutes at 37°C followed by three PBS washes. Meanwhile, cells were harvested with trypsin, washed with PBS and resuspended at $5.0x10^4$ cells/ml in 0.1% BSA/IMEM. The cell suspension was incubated for 1 hour at 37°C before adding to the precoated wells and incubated for 1 hour at 37°C to allow for adherence. The supernatant containing unattached cells was then removed, and the attached cells were stained with 50 μl of 0.05% crystal

violet (Sigma, St. Louis, MO) in 25% methanol for 15 minutes. The plate was gently rinsed by immersion in water and allowed to dry at room temperature. The incorporated dye was dissolved in 100 µl/well of 0.1M sodium citrate (Sigma) in 50% ethanol and measured by reading absorbance at 540 nm with a Dynatec MR700 ELISA reader (Dynatech, Chantilly, VA).

Transfection by electroporation of BSP DNA. The BSP sense vector was prepared by removing the full length transcript (1165 bp) from the EcoRI site of the Stratagene pBLUESCRIPT KS vector, B65g [31] blunting with Klenow, and cloning into the EcoRI site of the pcDNA3.1/Zeo mammalian expression vector (Invitrogen, San Diego, CA). Expression in this vector is under the control of an CMV enhancer-promotor and origin of replication to provide for high-level, constitutive expression and replication in mammalian cells, and SV40 polyadenylation signal provides for efficient processing of transcripts. An SV40-driven zeocin resistance cassette provides for the selection of stable transfectants (Figure 1). The MDA-MB-231BAG cell line was harvested with trypsin, washed twice in 1X PBS and suspended at $1X10^6$ cells/600 µl serum-supplemented IMEM medium. 5µg pcDNA3.1/ZeoBSP or empty pcDNA3.1Zeo vector was resuspended in 200 µl 1X PBS and mixed with the cells in an electroporation cuvette (BioRad, Hercules, CA). Cell transfection was carried out by electroporation using a Gene Pulser (BioRad) set at 960 µF and 250 V. Following electroporation, cells were incubated on ice for 10 minutes, then resuspended in 10 ml IMEM supplemented with 10% FBS and allowed to recover for 48 hours, after which the selective media was added (IMEM/FBS with 800 µg/ml Zeocin). Single cell clones were picked in the following weeks and screened for the production of BSP by Northern and Western analysis. Resultant clones were screened for BSP expression by Northern and Western analysis.

LCC15-MB Patient History/Cell Line Isolation. The patient presented at age 29 with a 2.5 cm mass in the right breast, which was biopsied, diagnosed as infiltrating ductal carcinoma, and removed along with 23 lymph nodes by modified radical mastectomy. The diagnosis was confirmed by mastectomy, and one of the 23 nodes found to contain metastasis. An apparently unrelated primary lesion arose in the left breast approximately 11 months later, was biopsied, and subsequently removed by modified radical mastectomy. Approximately 3 years after the initial primary tumor was

diagnosed, the patient presented with acute bone metastasis and was stabilized by hip replacement. Material from the bone metastasis was archived in paraffin, and processed for propagation in culture as the LCC15-MB cell line. To our knowledge, the patient did not present with metastases to any additional organs, and died from bone complications of the disease.

The LCC15-MB cells used here were between passages 20 and 30, and have been confirmed as a unique cell line derived from a human female by chromosomal analysis and characterization of isozyme phenotypes (Children's Hospital of Michigan, Detroit, MI). Other established human breast cancer (HBC) cell lines used in comparison (T47D, MCF-7_{ADR}, SKBr3, MDA-MB-453, BT549, MDA-MB-436, MDA-MB-231, MDA-MB-435, Hs578T) were originally obtained from the ATCC (Rockville, MD). MCF-7 cells were originally obtained from Dr. Marvin Rich (Michigan Cancer Foundation). All HBC cells were provided by the Lombardi Cancer Center Cell Culture Shared Resource and the UMR-106-01BSP rat osteosarcoma cell line was obtained from Dr. Larry Fisher (NIDR, NIH, Bethesda, MD). All cell lines were grown in Richter's Improved Minimal Essential Medium (IMEM, Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Gaithersburg, MD) and maintained at 37°C in 5% CO_2, 95% O_2. Cultures were confirmed as Mycoplasma free by the Lombardi Cancer Center Cell Culture Shared Resource using the Genprobe kit (Gen-Probe, San Diego, CA).

Matrigel and Collagen Outgrowth Assays. These assays were performed essentially as described previously [17,18]. For Matrigel outgrowth, 24 well tissue culture plates (Costar, Cambridge, MA) were coated with 300 µl Matrigel (a gift from Dr. Hynda Kleinman, NIDR, NIH) which was allowed to polymerize for 30 minutes at 37°C, after which time $1x10^5$ cells (total volume = 100 µl) in 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in IMEM (BSA/IMEM) were plated on top of the gel. Following an incubation of 1 hour to allow the cells to attach, the media was removed and another 250 µl of Matrigel layered over the cells resulting in a "sandwich" configuration. After a 30 minute incubation to allow gel polymerization, 500 µl of 10% FBS/IMEM was added to support growth of the cells. Collagen outgrowth assays were performed similarly,

except that a gel of fibrillar collagen (Vitrogen, Palo Alto, CA) was used in place of Matrigel. The cells were photographed at 40X magnification on a Zeiss IM-35 phase contrast microscope 3 days after cell plating.

Exogenous Gelatinize A (M.P.-2) Activation. LCC-15 MB cells were compared with MDA-MB-231, MDA-MB-435 and MCF-7 HBC cells for M.P.-2 activation analysis using previously established techniques [32,33]. Cells were plated in 24 well culture dishes (100,000 cells/well) for 24 hours in 10% FBS/IMEM. The next day, the cells were washed twice with unsupplemented IMEM and changed to serum-free medium (SFM; IMEM supplemented with 1mM sodium pyruvate, 2 mM glutamine, 1% nonessential amino acids, 1% trace elements, 1% HEPES buffer (Biofluids, Rockville, MD), and 1% ITS (Collaborative Biomedical Products) containing latent M.P.-2 (25% of SFM conditioned for 72 hours by M.P.-2-transfected MCF-7 cells; [34]). LCC15-MB cells were incubated with SFM/M.P.-2 in the presence or absence of Concanavalin A (Con A, 25 μg/ml, Sigma Chemicals, St. Louis, MO), clarified of cellular debris by centrifugation (2000 rpm, 20 minutes) after 24 hours, and analyzed for M.P.-2 activation with gelatin zymography.

B. Results and Discussion

1. Expression of bone sialoprotein in human breast cancer

a. Localization of BSP to primary breast tumors and their corresponding bone metastases

In order to confirm recent studies which showed BSP to be expressed in a majority of primary breast lesions surveyed, we screened several preserved, archival specimens from the Lombardi Cancer Center tumor bank by immunocytochemistry. As expected, there was positive BSP staining in all five samples surveyed. Two representative specimens are shown in Figures 2A and 2B, in which tumor cells appeared to be stained a dark-brown color, while the surrounding mammary stroma remains unstained. Furthermore, since BSP is normally localized in the bone matrix, it is thought that expression of BSP by primary tumor cells may help to promote bone-specific metastasis. Consequently, we also examined breast tumor material from patients who later developed bone complications, and showed positive BSP staining in both the primary tumor cells and in resultant bone metastases (Figures 2C and 2D). Although it is synthesized and present in bone matrix, the majority of BSP in this case, seems to be associated with the tumor cells.

b. BSP is not expressed by human breast cancer cell lines *in vitro* or *in vivo*

We were interested in studying more closely the role of BSP in breast cancer invasion and metastasis using HBC cell lines. Therefore, we assayed a range of HBC cell lines, from the least invasive, estrogen and progesterone receptor positive MCF-7 cells [28] to the more traditionally invasive and metastatic MDA-MB-435 cells [17], and found, surprisingly, that no cell line substantially expressed BSP. Northern analysis (Figure 3A) of the cell lines revealed a lack of BSP message in all but the UMR-106-01 BSP (UMR) rat osteosarcoma cell line, employed as a positive control for the bone matrix proteins [35]. GAPDH was used as a loading control for the Northern blots (Figure 3A). Similarly, Western analysis of several of the same HBC cell lines also failed to reveal BSP protein (approximately 70 kD) in all but the UMR-106-01 BSP cell line (Figure 3C). A dialyzed extract of bone matrix proteins obtained during hip replacement surgery may also contain BSP, although apparently high levels interfere with discrimination of a band at 75kD. Finally, an immunocytochemical analysis of several paraffin-embedded cultured cell plugs (Figure 4A-D) also

confirmed the absence of BSP expression in the HBC cell lines. Again, the UMR cell line was used as a positive control for BSP and displayed positive cytoplasmic staining.

In order to exclude the possibility that BSP expression was turned off or downregulated *in vitro*, we extracted and analyzed BSP RNA from three HBC cell lines grown as nude mouse xenografts. As shown in Figures 3B and 4E-H, only the UMR tumor xenograft expressed BSP message and stained positively for BSP by immunocytochemistry, indicating against the possibility that BSP was downregulated in culture.

Overall, our expression studies are confounding, because while BSP is present in primary and metastatic tumors, it appears to be absent in cultured breast cancer cell lines. A recent report demonstrated very low levels of BSP expression by a few HBC cell lines as assayed by RT-PCR, but it is unlikely that this small amount derived from HBC cells could be responsible for the levels seen in primary and metastatic specimens. An *in vivo* induction of BSP by other tumor-associated factors is possible, and would presumably raise BSP levels in the tumor. Furthermore, if BSP is preferentially expressed by those primary tumors which are bone-metastatic, as suggested by another studies from Castronovo's lab [5], the absence of the protein in cultured cell lines would not be totally unexpected. In fact, none of the currently available breast cancer cell lines were derived from a bone metastasis. Most were derived from pleural effusions [36,37], although BT-20 cells were derived from a primary mammary adenocarcinoma [38], Hs578T from a primary mammary carcinosarcoma [38], and the MDA-MB-361 from a brain metastasis [39]. Nonetheless, the LCC15-MB cell line recently isolated from a breast-bone metastasis also lacked BSP expression *in vitro* and *in vivo*, despite its bone metastatic derivation. It is also possible that the BSP protein is temporally modulated, being expressed, for example, only during a narrow window of time when tumor cells are correctly poised to leave the primary tumor and metastasize to a secondary site. Although the widespread expression of BSP in primary tumors argues against this paradigm, BSP expression could already be downregulated at time of cultured cell line isolation. Finally, recent *in situ* hybridization studies showed that the related bone matrix protein osteopontin, (OPN), was expressed primarily by tumor associated macrophages in a number of different primary cancers including

breast tumors [40,41]. High levels of secreted OPN were adsorbed to the adjacent tumor cells, giving the appearance that it was expressed by tumor cells in immunocytochemical studies. Needless to say, similar *in situ* studies investigating the cellular origin of BSP in both primary lesions and bone metastases will be necessary to clarify these expression studies.

c. Expression of BSP in the MDA-MB-231BAG HBC cell line

Since BSP was not present in detectable levels in the established HBC cell lines examined, we transfected BSP cDNA into the MDA-MB-231BAG HBC cell line, which produces no endogenous BSP. These cells have been transduced with a retrovirus encoding the Lac Z gene which renders the cells blue upon staining with X Gal and thus allows for simple detection of the cells in various assays [27]. MDA-MB-231BAG cells were transfected with the pcDNA3.1/ZeoBSP construct (Methods) or the vector alone. This particular mammalian expression vector contains the cytomegalovirus immediate early promotor which drives expression of the BSP transcript represented by a full length BSP cDNA, as well as the Zeo gene which confers resistance to the toxic effects of the antibiotic, Zeocin (Invitrogen, San Diego, CA). Transfected cells containing an integrated copy of the BSP construct were selected by passaging in culture medium containing 800 μg/ml Zeocin, and after approximately weeks, 30 transfected colonies were isolated and the rest pooled. A similar number of vector control colonies, transfected with the empty pcDNA3.1/Zeo cassette were also isolated and the remainder pooled.

Northern blot analysis of the transfectants revealed BSP expression in 5 clones and 2 pooled populations, while vector controls and the parental cell line were negative for BSP message (Figure 5A-C). The UMR rat osteosarcoma cell line was used as a positive control. Interestingly, the message from BSP-transfected cells migrated at a single band, different from the 2- banded transcript produced by the UMR-106-01 cell line. However, the sizes of the transcripts did not vary noticeably, and the discrepancy observed is most likely due to the absence of a natural form of BSP (the larger sized band in the UMR cells) in the transfected MDA-MB-231 BAG cells, which normally do not produce any BSP. GAPDH was again used as a positive loading control for Northern analysis. Western analysis of these clones was also completed (Figure 5D), confirming

the presence of a 75 kD BSP protein in our selected clones. The BSP protein was represented by two distinct bands in most of the BSP clones, and additionally, the ratio of the two bands differed from one transfectant to another, possibly depending on variations in posttranslational modifications between species (UMR-106-01 BSP vs. MDA-MB-231) and cell populations. In comparing the Northern and Western data, a certain extent of correlation can be seen between BSP RNA and protein levels, although clones appearing to express high levels of RNA did not always produce high protein levels.

d. BSP-expression in MDA-MB-231BAG results in increased migration and diminished adhesion *in vitro*

After verifying BSP expression in our transfected cells, we examined some of the *in vitro* cellular characteristics associated with invasion and metastasis. Cellular migration represents one of the important components of the metastatic cascade, occurring when tumor cells have dissociated from the primary mass and are moving toward the bloodstream as well as after extravasation, as they migrate to a secondary, metastatic site. We employed the Boyden Chamber assay to measure cellular migration levels in parental MDA-MB-231BAG cells as compared to the transfectants which expressed BSP. When serum-supplemented medium was used as the chemoattractant, all cells migrated readily across the filter. However, with serum-free medium as the chemoattractant, the BSP-transfected cells most effectively crossed the porous barrier (Figure 6A).

Cellular adhesion is also an important part of the metastatic process, and differential adhesion molecules have been seen in various breast cancer cell lines [42]. We compared the adhesion profile of the BSP transfectants on plastic, vitronectin and collagen Type I to parental MDA-MB-231BAG cells as well as vector-transfected cell lines (as shown in Chapter 3, these HBC cells express the vitronectin-binding $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins). Parental MDA-MB-231BAG and vector control cells adhered well to all three substrates, while BSP transfectants attached equally as well to Type I collagen, but appeared to adhere less well to either plastic and vitronectin (Figure 6B). This suggests that the endogenous BSP produced by the transfectants may be interfering with adhesion receptors which usually mediate attachment to these particular matrices. This perturbance or

differential usage of adhesion receptors would be important in allowing a population of tumor cells to detach from the primary tumor, metastasize, and re-attach to a new secondary site of growth.

Our results demonstrating that breast cancer cell-derived BSP could effectively increase overall migration and selectively decrease adhesion, support an autocrine model of action at the primary tumor site, in which BSP produced by the tumor cell acts upon itself to potentiate invasive behavior. This type of autocrine action, where tumor malignancy and invasion are correlated with endogenous production of various growth factors, proteinases, angiogenesis factors, and motility factors was the topic of a recent review [3]. On the other hand, a paracrine mode of action may also exist, and similar to OPN, BSP could be produced by non-malignant tumor-associated cells in a primary lesion. The paracrine mechanism may also predominate in the bone environment, where BSP is already abundant and tumor cells need not produce endogenous BSP in order to maintain a metastatic phenotype.

2. The LCC15-MB human breast cancer cell line expresses osteopontin and exhibits an invasive and metastatic phenotype

a. LCC-15 cells display an invasive phenotype *in vitro*

We have recently reported that LCC15-MB cells express the mesenchymal intermediate filament protein vimentin [16]. Vimentin expression in carcinoma-derived cells is thought to arise through a form of the epithelial to mesenchymal transition (EMT, reviewed in [19]), a transformation seen in epithelial cells when migration is required. Since we have associated vimentin expression with *in vitro* invasiveness in HBC cell lines [17,19], we examined the LCC15-MB cells here in a variety of invasion assays, including the Matrigel- and collagen-outgrowth assays, and Boyden chamber assays for chemoinvasion and chemomigration.

As seen in Figure 7, LCC15-MB cells show a stellate morphology on plastic compared to the relatively polygonal, epithelial clusters adopted by the MCF-7 cells, and are thus comparable to the invasive MDA-MB-231 HBC cell line. When tested in the Matrigel outgrowth assay, the LCC15-

MB cells formed stellate, penetrating colonies as seen with MDA-MB-231 cells, while the MCF-7 cells formed regular spheroidal colonies. In the collagen outgrowth analysis, LCC15-MB cells appeared the most stellate and invasive, although the MDA-MB-231 cells also formed protrusive colonies. In contrast, the MCF-7 cells formed largely spheroidal colonies in collagen, similar to their morphology in Matrigel (Figure 7). These data indicate that the LCC15-MB cells resemble the more invasive HBC cells lines in terms of their morphological responses to extracellular matrix.

We examined this further using Boyden chamber assays for chemoinvasion and chemomigration [17,18]. As shown in Figure 8A, LCC15-MB cells showed levels of chemoinvasion through a Matrigel barrier intermediate between MDA-MB-231 and MCF-7 when 10% FBS was used as a chemoattractant. Intrinsic chemoinvasion without a chemoattractant (0.1% BSA) was not demonstrated by LCC15-MB cells, however, and was only seen in the MDA-MB-231 cell line. Preliminary analyses with various other vimentin-positive HBC cells have shown that MDA-MB-231 cells are quite unusual in this regard, since all other cell lines tested required a chemoattractant (NIH-3T3 fibroblast cell-conditioned media was employed; Torri and Thompson, unpublished). Chemomigration over a collagen-coated filter designed not to present a barrier (chemotaxis; [17,43]), and employing 10% FBS as a chemoattractant also showed a relatively low level of migration for LCC15-MB cells (Figure 8B).

The preferential activity of the LCC15-MB cells for chemoinvasion rather than chemotaxis suggests that they may have some advantage in matrix degradation. A unifying feature of the vimentin-positive, invasive HBC cells is the ability to activate MMP-2, the 72 kD gelatinize A implicated in basement membrane invasion by virtue of its ability to cleave type IV collagen found in basement membranes [44,45]. Such activation is not constitutive, and can be induced by treatment with gels of fibrillar collagen [46,47] or more rapidly and potently with the plant lectin Concanavalin A (ConA, [32]). Like the invasive, vimentin-positive MDA-MB-231 and MDA-MB-435 cell lines, LCC15-MB cells showed activation of exogenous MMP-2 after ConA treatment, while the MCF-7 vimentin-negative cell was unable to activate MMP-2 (data not shown).

b. Attachment profile of LCC15-MB cells

Cellular adhesion represents an important step in the metastatic process, and differential expression of adhesion molecules has been seen in various human breast cancer cell lines [42]. We compared the adhesion profile of the LCC15-MB cells on various substrates to the MDA-MB-231, MDA-MB-435 and MCF-7 HBC cell lines. As shown in Figure 9, adhesion of the LCC15-MB cells to the different substrates was comparable to that of the MDA-MB-231 cells, falling between the highly adhesive MCF-7 cells and the non-adhesive MDA-MB-435 cells. These adhesion profiles were previously studied by Maemura et al. [48], and appear to correlate inversely with the cell line's level of metastasis. Comparing the adhesion of HBC cells to specific substrates suggests that there is also a specificity in the adhesion phenotype. For example, although MDA-MB-231 cells attach very well to collagen IV and Matrigel, the LCC15-MB cell line attaches best to vitronectin while also adhering to Matrigel. It is interesting to note that the LCC15-MB cells attach relatively poorly to type IV collagen compared to the other breast cancer cell lines and substrates.

c. Osteopontin expression by LCC15-MB cells

Osteopontin expression has been recently implicated in the metastatic capacity of breast carcinoma [20], so we examined OPN expression by LCC15-MB cells in comparison to other HBC cell lines at the mRNA and protein levels. Interestingly, Northern analysis (Figure 10A) revealed OPN mRNA expression only in the LCC15-MB and highly metastatic MDA-MB-435 cell lines [17,26,49,50]. The UMR-106-01BSP rat osteosarcoma cell line was employed as a positive control for OPN [35]. Similarly, Western analysis (Figure 10C) with the LF19 polyclonal antibody to OPN revealed expression of an approximately 75 kD band in these two cell lines, but not in a number of other cell lines including MCF-7 and MDA-MB-231. Immunocytochemical analysis of paraffin-embedded cultured cell plugs (Figure 11) showed retention of OPN expression patterns in the UMR-106-01BSP cells as well as in the MDA-MB-435 and LCC15-MB cell lines. In contrast to these high levels of OPN expression, the protein appeared to be absent in the MDA-MB-231 cells. In order to examine *in vivo* OPN expression, these four representative cell lines were injected subcutaneously into nude mice, where OPN protein was maintained in all three cell lines and again not seen in MDA-MB-231 (Figure 10B).

The observation that LCC15-MB cells express OPN prompted us to examine OPN expression in archival paraffin-embedded specimens from the original primary tumor and the bone metastasis from which the LCC15-MB cell line was derived (Figure 12). We also examined a lymph node metastasis associated with the original tumor and a ductal carcinoma *in situ* (DCIS) which arose in the ipsilateral (left) breast one year after the removal of the original lesion by modified radical mastectomy. These specimens have been detailed more completely in the original description of the LCC15-MB cells [16]. It was interesting to find that OPN was abundantly detected in the invasive, right primary mammary lesion and the right side lymph node metastasis as well as in the benign DCIS on the left (Figure 12). OPN levels appeared especially high in the bone metastasis, where bone-derived OPN may contribute to overall staining through adsorption.

d. Expression of the bone matrix protein osteopontin in LCC15-MB xenografts

We further examined the expression of OPN in LCC15-MB cells grown as either a primary tumor xenograft or as an arterially-seeded bone metastasis in nude mice (Figure 13). LCC15-MB cells were subcutaneously implanted or injected *i.c.* as described in the Materials and Methods. Upon subcutaneous inoculation into the mammary fat pad region, the LCC15-MB cells showed exponential growth after a lag phase of 7 days. The tumors arose in 100% of mice inoculated, appearing to be homogeneous, poorly differentiated carcinomas (Figure 13A) with a doubling time of approximately 6±2 days. We detected no distant soft organ or bone metastases in any of the subcutaneously injected animals as assayed by routine histology.

Since LCC15-MB cells were derived from a bone metastasis, we also used a recently described model for bone metastasis analysis in nude mice [27,51,52]. LCC15-MB cells were injected *i.c.* in two separate experiments. In the first experiment, target organs were minced and cultured, and the metastasis to different organs scored on the basis of LCC15-MB cell outgrowth. LCC15-MB cells formed occasional lesions in a number of organs (kidney, spleen, lung, liver, heart and brain), but consistently colonized bone, as assessed by the outgrowth from cultured organs or bones resected from each mouse (16/22 cultures). One mouse developed an intraperitoneal tumor which may have been seeded through injection error. In the second experiment, we processed bone, spleen, kidney,

brain, lung and liver for histological analysis four weeks after *i.c.* injection. Histologically evident bone metastasis was apparent in one of the four bones sectioned, as illustrated in Figure 13C. These data confirm the capability and propensity of this cell line for bone metastasis. The nature of the bone metastases was difficult to determine by histology; however, the lesions did not appear to be blastic. In fact, preliminary evaluation of the bones by radiography suggests that LCC15-MB metastases are potentially lytic, not surprising, given the lytic nature of the tumor from which the LCC15-MB cell line was derived.

Importantly, we found that in addition to *in vivo* tumorigenicity and propensity for bone colonization, LCC15-MB breast cancer cells retain OPN expression *in vivo*, as indicated by cytoplasmic staining in at least 90% of the carcinoma cells as well as in the interstitial stroma of the subcutaneous nude mouse xenografts (Figure 13B). Similarly, in Figure 13D, tumor cells metastasizing to and colonizing the bone marrow cavity of the animal also stained positively for OPN. In the mouse bone, there is faint immunostaining in the bone matrix, but staining is absent in the hematopoietic component of the marrow.

These LCC15-MB studies have contributed to the development of a new cellular model for the study of metastasis from human breast cancer in general, and particularly to bone. The LCC15-MB cells are shown to be distinct from other human breast cancer cell lines genetically, but have a mesenchymal-like phenotype indicated by vimentin expression, a hallmark shared by other metastatic cell lines. In addition to revealing an invasive morphology in Matrigel and type I collagen, these vimentin-positive cells adhere poorly to various matrices, an reveal an invasive phenotype as measured by the Boyden chamber assay, and the capacity to activate MMP-2. Comparable to other HBC cell lines, the expression of vimentin by the LCC15-MB cells along with expression and/or activation of MMP-2, the 72-kDa type IV collagenase implicated in basement membrane degradation and cancer metastasis [19], are consistent with its derivations from an aggressively metastatic tumor. Furthermore, the LCC15-MB cell line also expressed the bone matrix protein osteopontin which was recently associated with breast cancer metastasis [20,53]. The expression of OPN by LCC15-MB cells was retained in our *in vivo* nude mouse models, both in the

primary xenograft and bone metastasis. OPN was also present in the primary mammary lesions and lymph node metastasis in archival patient specimens from which the LCC15-MB cell line was derived. Expression of OPN may thus contribute to the invasive nature of the LCC15-MB HBC cell line (increased migration and degradation characteristics), and could help to explain the bone metastatic pathway of these cells *in vivo*.

Although the precise mechanism(s) by which OPN could promote metastasis is unknown, it has been hypothesized that the RGD domain of osteopontin could interact with specific integrins expressed on the surface of tumor cells to facilitate attachment and migration of the cells to bone matrix and possibly proliferation, through integrin signaling mechanisms. Through its RGD integrin binding domain, OPN provides cell attachment capability for many cell types in the bone, including osteoblasts, osteoclasts and fibroblast cell lines (rev. in [22]). In addition, OPN has been shown to be associated with other bone extracellular matrix components including collagen I, fibronectin and osteocalcin, possibly acting as a bridge between cells and ECM in the bone [22]. Such interactions may also occur between the tumor cells and osseous structures. We recently observed a similar interaction between BSP and MDA-MB-231 cells, where the $\alpha v\beta 3$ integrin appeared to mediate migratory behavior of the cells, while the $\alpha v\beta 5$ integrin was necessary for both adhesive and proliferative effects [15]. *In vitro*, the vimentin-positive MDA-MB-435 breast cancer cells, which have been shown to express high levels of OPN, showed increased migration and adhesion to OPN, and this could be subsequently blocked by mutating the RGD sequence of the protein [21]. Interestingly, preliminary data from our laboratory indicate that migration of LCC15-MB and MDA-MB-435 HBC cells toward BSP is also integrin-dependent; however, these cell lines actually migrate less readily towards BSP than the MDA-MB-231 cells for example, which do not express either of the sialoproteins (unpublished data). It will be potentially interesting to examine LCC15-MB cell responses to OPN and to see whether these responses are altered due to endogenous production of OPN. The expression of OPN by the LCC15-MB cells may thus help to explain their original derivation from bone, as well as their preference for, and subsequent return to bone in our *in vivo* metastasis model.

C. Recommendations

The original statement of work submitted with this proposal listed five tasks, several of which we have already addressed (both in this and last year's annual report), and the remainder of which is work in progress:

Task 1. Examine bone sialoprotein (BSP) and osteopontin (OPN) mRNA and protein expression in human breast cancer (HBC) cell lines (*in vitro* as well as in different microenvironments *in vivo).*

a. We have examined BSP and OPN mRNA and protein expression in HBC cell lines *in vitro* and nude mouse xenografts *in vivo* (Northern and Western analyses completed). However, we could not compare BSP expression in bone metastatic cell sublines with those metastasizing to select soft organs since none of the HBC cell lines initially studied appear to express BSP.

b. BSP and OPN immunoreactivity in HBC cell xenografts and the LCC15-MB bone-derived HBC cell line has been addressed as well as immunocytochemical analysis of HBC cell lines, paraffin-embedded breast cancer specimens and associated bone material. A more detailed study of the LCC15-MB cell line has also been completed. This study examined BSP and OPN expression by the cell line as well as primary tumor, lymph node metastasis and bone metastasis from which it was derived in order to better characterize the molecular mechanisms of bone metastasis and to explore possible correlations between bone metastasis and the expression of these sialoproteins. In addition, this study demonstrated the *in vivo* metastatic potential of the LCC15-MB cell line.

Task 2. Examine migratory and proliferative responses of HBC cells to BSP and OPN protein and peptides.

a. We have completed migration analysis of HBC cell lines toward BSP protein, recombinant BSP fragments and BSP-derived RGD peptides (reviewed in depth in the previous annual report) using the Boyden chamber technique. We are continuing to examine the migration responses of other HBC cell lines. Although OPN and several recombinant fragments (but not peptides) have been made available to us, similar work appears to be in progress at another laboratory, and for these

reasons, we chose to focus mostly on cellular responses to BSP and to investigate in detail, the domains of the protein necessary for these responses.

b. We have completed proliferation analysis of MDA-MB-231 cells towards BSP using 96 well plate assays and have also begun to examine several other cell lines. Again, we chose to focus on BSP responses instead of pursuing similar experiments with OPN. Although not mentioned in the original tasks, we have completed additional studies examining *in vitro* cell adhesion of the MDA-MB-231 cells to BSP, BSP fragments and peptides.

Task 3. Investigate *in vivo* metastatic ability of HBC cells expressing BSP/OPN, especially those which are bone-derived.

a. As addressed above, we did not find any HBC cell lines which expressed BSP (*in vivo* or *in vitro*), making it difficult to rationalize potential animal studies. However, the LCC15-MB cell line does express the closely related OPN, and we have shown that these cells are bone metastatic *in vivo* in nude mice, as well as possessing characteristics consistent with other invasive HBC cell lines. There are also plans to test BSP transfectants in *in vivo* metastasis assays.

Task 4. Transfect cells with sense or antisense vectors to further examine the role of BSP and OPN in *in vivo* metastasis.

a. Based on our findings which show lack of BSP expression in breast cancer cell lines, we have expressed a BSP sense vector in the MDA-MB-231 HBC cell line which does not endogenously express BSP. Following transfection of BSP, we selected a number of BSP-expressing clones and have screened for BSP expression by both Northern (RNA) and Western (protein) analysis. These clones were tested in *in vitro* migration and adhesion assays in order to determine whether or not BSP may play a role in invasion of HBC cells. We will also examine our transfectants *in vivo* (both tumor xenograft and intracardiac injection models) to determine if BSP expression increases tumor growth and invasion in a nude mouse model.

Task 5. Elucidate the domains of these proteins responsible for increased migration and proliferative potential, as well as increased *in vivo* bone metastasis.

a. After determining positive migratory, proliferative and adhesive responses of HBC cells to BSP, we used recombinant BSP fragments and the BSP-derived RGD peptides to elucidate the domain of the protein responsible for these behaviors (the RGD integrin-binding domain).

b. Because we determined the RGD domain to be responsible for migration, proliferation and adhesion responses, we used integrin-blocking antibodies, rather than peptides, to try and block the BSP-induced responses of the HBC cell line. After identifying specific antibodies which blocked these responses, we also selected for subpopulations of the cell line which showed augmented responses depending on augmented expression of a specific integrin. We are currently testing whether selectively increased expression of a certain integrin will potentiate bone vs. soft organ metastasis *in vivo*.

V. Conclusions

Osteolytic metastasis affects approximately two-thirds of women with breast cancer, and is one of the leading causes of death in a significant number of patients (rev. in [3]). Although it is a selective process, the precise mechanisms responsible for tumor spread to the bone are little understood. Many have demonstrated the importance of tumor cell-ECM interactions in the process of metastasis, showing that these interactions are as critical as inherent properties of the tumor cell. For example, characterization of the newly established LCC15-MB bone metastatic HBC cells showed varied morphology depending on the type of matrix on which the cell line was grown. We have studied the interactions between HBC cells and BSP, a bone matrix protein, and demonstrated that BSP directly stimulates these cells by influencing proliferative, migratory and adhesive properties of HBC cells, through interactions with specific integrins on the tumor cell surface. Recent immunocytochemical studies have described the presence of BSP in about 80% of primary breast lesions, and have correlated BSP immunoreactivity with aberrant microcalcifications, decreased patient survival and the development of bone metastasis [5,7,8]. We also localized BSP immunostaining to primary breast cancer specimens, as well as to bone metastases, strengthening earlier findings that BSP may support HBC cell colonization in bone. However, our findings also suggest that BSP may be responsible for influencing malignant progression at the primary tumor. Failure to detect BSP RNA by Northern analysis of a number of HBC cell lines suggests that non-tumor cells may also be a source of BSP in primary breast cancers, while osteoblasts and osteoclasts are a source of BSP in the bone environment. This does not rule out the possibility for an autocrine mode of action, especially since HBC cells engineered to express BSP showed increased migration and decreased adhesion, both contributing to increased tumor invasivity. *In vivo* experiments using a bone metastasis/PCR quantitation model will help to determine if BSP expression by HBC helps to promote metastasis, and whether this metastasis is specifically directed toward bone. Additional studies with integrin selectants or other molecular perturbations may indicate which BSP-induced biological responses are most important in tumor invasion. Because bone metastasis is a complex event, it is unlikely that BSP acts alone in mediating breast cancer invasion and metastasis. However, since BSP is present in breast cancer lesions, and does seem to contribute to increased invasive behavior of breast tumor cells, elucidation of the interactions occurring between HBC cells and BSP may eventually result in

a better understanding of breast cancer progression and possibly, new and more effective clinical treatments for metastatic disease.

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VII. Appendices

Figure 1

Schematic diagram of the pcDNA3.1/Zeo/BSP expression vector. The full length BSP transcript (1165 bp) was removed from the EcoRI restriction site of B65g plasmid (in pBLUESCRIPT) and cloned into the pcDNA3.1/Zeo mammalian expression vector.

Figure 2

Immunohistochemical analysis of BSP in two primary breast tumor specimens **(A,B)** and a primary breast tumor with its associated bone metastasis **(C,D)** as detected by polyclonal antibody LF 83. All archival paraffin-embedded tissue was from the Lombardi Cancer Center Tumor Bank. Magnification = 10X.

Figure 3

Expression of BSP mRNA in HBC cell lines **(A)** and nude mouse xenografts **(B)** as assayed by Northern and Western blotting **(C)**. **A:** Lane 1-MCF-7, 2-T47D, 3-MCF-7_{ADR}, 4- SKBr3, 5-MDA-MB-453, 6- BT549, 7- MDA-MB-436, 8-MDA-MB-231, 9-MDA-MB-435, 10- LCC15-MB, 11-Hs578T, 12- UMR-106-01BSP. **B:** Lane 1,2: UMR-106-01BSP, 3,4-LCC15-MB, 5,6-MDA-MB-435, 7,8- MDA-MB-231. RNA loading was normalized using a GAPDH control. **(C)** BSP protein expression in HBC cell lines was analyzed by Western analysis using the polyclonal antisera LF100, revealing a band at approximately 75 kD. Lane 1-MCF-7, 2-MDA-MB-231 BAG, 3-MDA-MB-231, 4-bone-derived extract, 5- MDA-MB-435, 6- LCC15-MB, 7-UMR-106-01 BSP. In both the Northerns and Western, the UMR-106-01BSP rat osteosarcoma cell line which expresses endogenous BSP was used as a positive control.

Figure 4

Immunocytochemical analysis of BSP in paraffin-embedded, scraped cell plugs **(A-D)** and nude mouse xenografts **(E-H)** of the rat osteosarcoma cell line UMR-106-01BSP **(A,E)**, and HBC cell lines MDA-MB-231 **(B,F)**, LCC15-MB **(C,G)** and MDA-MB-435 **(D,H)** as detected by a polyclonal

antibody to BSP (LF83). Magnification = 40X.

Figure 5

RNA **(A-C)** and protein **(D)** analysis of BSP in transfected MDA-MB-231 clones, vector transfected controls and parental cells. Northern blots **(A)** Lanes 1-5: vector controls, 6-8: BSP clones *15, 8* and *16*, 9: MDA-MB-231BAG, 10-11: BSP clones *1* and *3*, 12: UMR-106-01BSP. **(B)** Lanes 1-5: vector controls, 6: MDA-MB-231BAG, 7-9: BSP clone *12, pool 2* and clone *1*, 10-11: UMR-106-01BSP. **(C)** Lanes 1-7: BSP clones *1* and *3*, *pools 1 and 2*, clones *15, 4*, and *6*, respectively, 8: MDA-MB-231BAG, 9-11: vector controls, 12: UMR-106-01BSP. Top panels of Northerns show BSP message while lower panels depict GAPDH gel loading controls. **(D)** Western blots (BSP polyclonal antibody LF100), Lane 1: BSP clone *15*, 2- 3: vector controls, 4: MDA-MB-231BAG, 5: vector control, 6: BSP clone 6, 7: BSP *pool 2*, 8:BSP *pool 1*, 9: BSP clone *1*, 10 and 11: molecular weight markers, 12: vector control, 13: BSP clone *3*, 14: BSP clone *6*, 15: BSP clone *12*, 16: BSP clone *1*, 17: UMR-106-01 BSP, 18: MDA-MB-231BAG, 19: LCC15-MB, 20: vector control.

Figure 6

BSP-transfected MDA-MB-231 cell migration **(A)** to serum-free media as compared to vector control cells (10% serum-supplemented media (FBS) was used as a positive control) and attachment **(B)** to PL (plastic), VN (vitronectin), and Type I collagen. Bars represent mean and standard error from triplicate filters/wells in a representative experiment. * $P<0.05$.

Figure 7

Morphology (phase contrast microscopy) of the LCC15-MB **(A,B,C)**, MDA-MB-231 **(D,E,F)** and MCF-7 **(G,H,I)** cell lines cultured on plastic **(A,D,G)**, on a 3-dimensional gel of Matrigel **(B,E,H)**, and on a gel of fibrillar collagen **(G,F,I)**. Compare the stellate morphology of the MDA-MB-231 and LCC15-MB cells and their invasive phenotype on Matrigel to the compact morphology and non-invasive colonies of MCF-7 cells on plastic and Matrigel, respectively. Magnification = 40X.

Figure 8

Boyden chamber chemoinvasion **(A)** and chemomigration **(B)** of the LCC15-MB, MDA-MB-231 and MCF-7 cell lines toward either bovine serum albumin (BSA, gray bars) or fetal bovine serum (FBS, black bars) as indicated. Boyden chamber activity is expressed as number of cells migrating across a filter per high power (40X) microscope field. Data are presented as the mean with standard error, and are representative experiments.

Figure 9

Adhesion profiles of LCC15-MB, MDA-MB-231, MDA-MB-435 and MCF-7 HBC cell lines to plastic, vitronectin, collagen IV, fibronectin, Matrigel and collagen I, as measured by absorbance at 540 nm.

Figure 10

Expression of OPN mRNA in LCC15-MB and various other HBC cell lines **(A)** and in nude mouse xenografts **(B)** as assayed by Northern blotting. **A:** Lane 1-MCF-7, 2-T47D, 3-MCF-7_{ADR}, 4-SKBr3, 5-MDA-MB-453, 6- BT549, 7- MDA-MB-436, 8-MDA-MB-231, 9-MDA-MB-435, 10-LCC15-MB, 11-Hs578T, 12- UMR-106-01BSP. **B:** Lane 1,2: UMR-106-01BSP, 5,6-LCC15-MB, 8,9-MDA-MB-435, 10- UMR. RNA loading was normalized using a GAPDH control. **(C)** OPN protein expression in HBC cell lines as detected by Western analysis (OPN antibody LF19), revealing a band at approximately 75 kD. Lane 1-empty, 2-UMR-106-01BSP, 3-empty, 4-MCF-7, 5- MDA-MB-231, 6- LCC15-MB, 7-MDA-MB-435. In both the Northerns and Western, the UMR-106-01BSP rat osteosarcoma cell line which expresses endogenous OPN was used as a positive control.

Figure 11

Immunocytochemical analysis of OPN in paraffin-embedded, scraped cell plugs of UMR-106-01BSP **(A,E)**, MDA-MB-231 **(B,F)**, MDA-MB-435 **(C,G)** and LCC15-MB **(D,H)** cell lines **(A-D)** and nude mouse xenografts **(E-H)** as assayed using the polyclonal antibody to OPN (LF19). Magnification = 40X.

Figure 12

Immunocytochemical analysis of OPN in the right primary, invasive breast carcinoma **(B)**, left breast DCIS **(D)**, right side lymph node metastasis **(F)** and corresponding bone metastasis **(H)** of the patient from which the LCC15-MB cell line was derived, accompanied by corresponding H&E stained specimens **(A,C,E,G)**. Magnification = 10X.

Figure 13

Immunohistochemistry of LCC15-MB subcutaneous xenograft **(A,B)** and bone metastasis **(C,D)** in the nude mouse stained with routine H&E **(A,C)**, or with LF19 for osteopontin **(B,D)**. Magnification = 10X.

Figure 1

pcDNA 3.1/Zeo-BSP

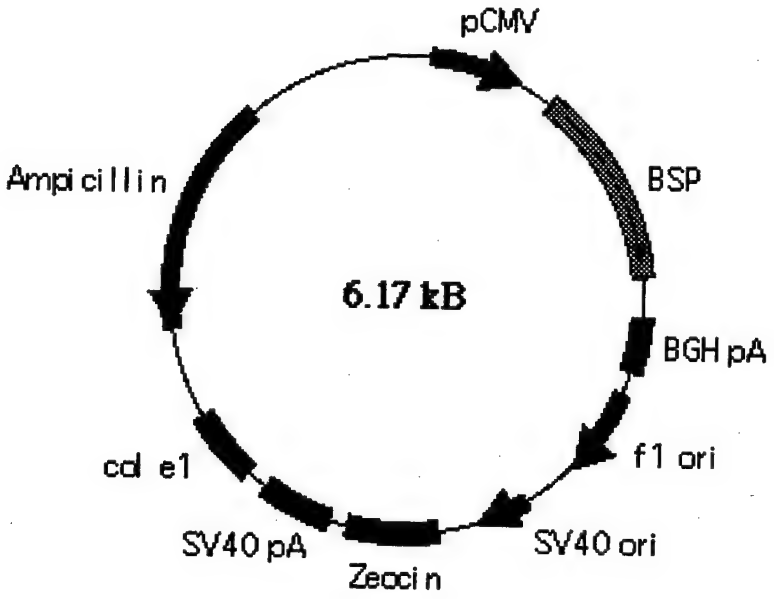
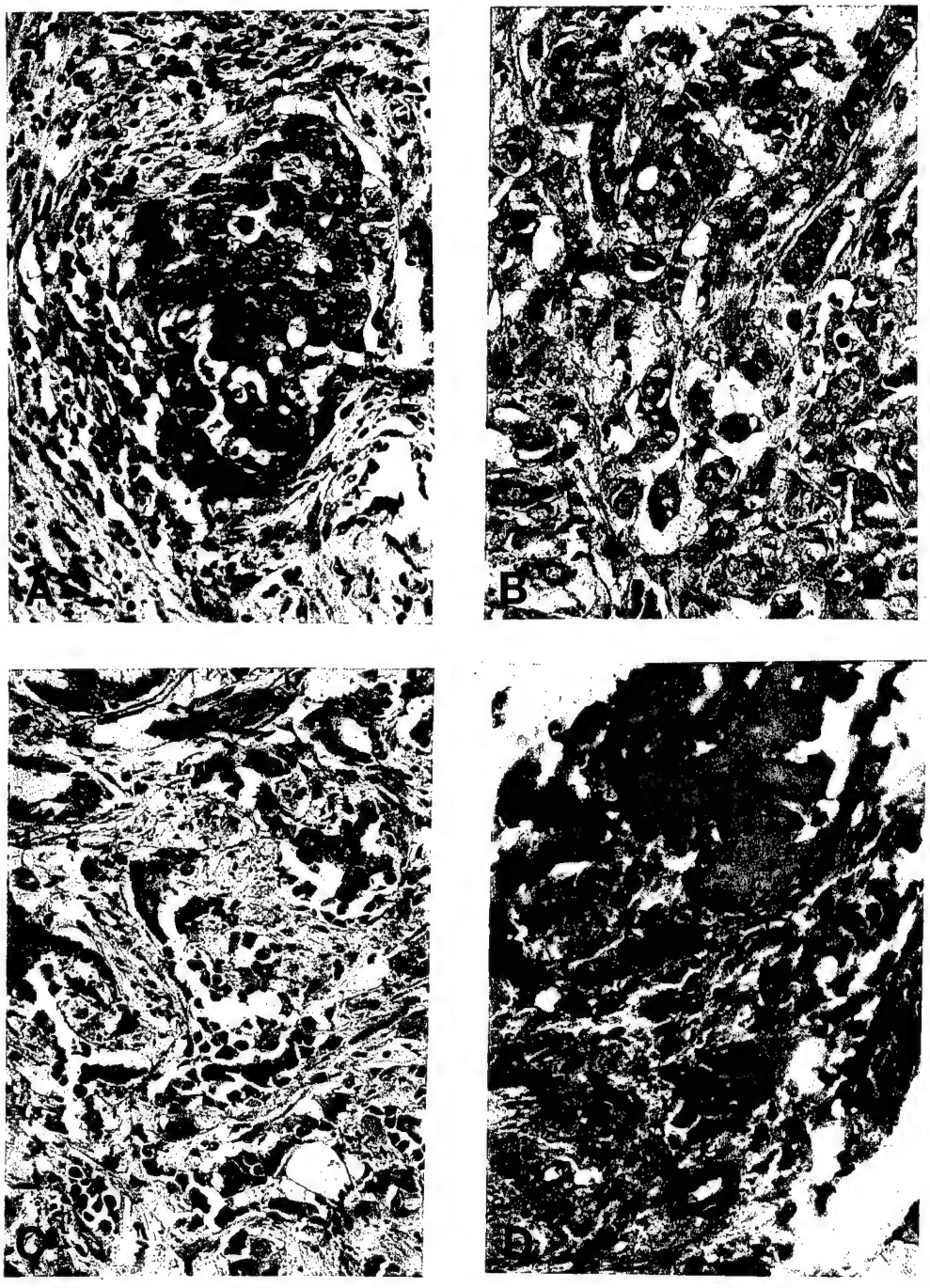
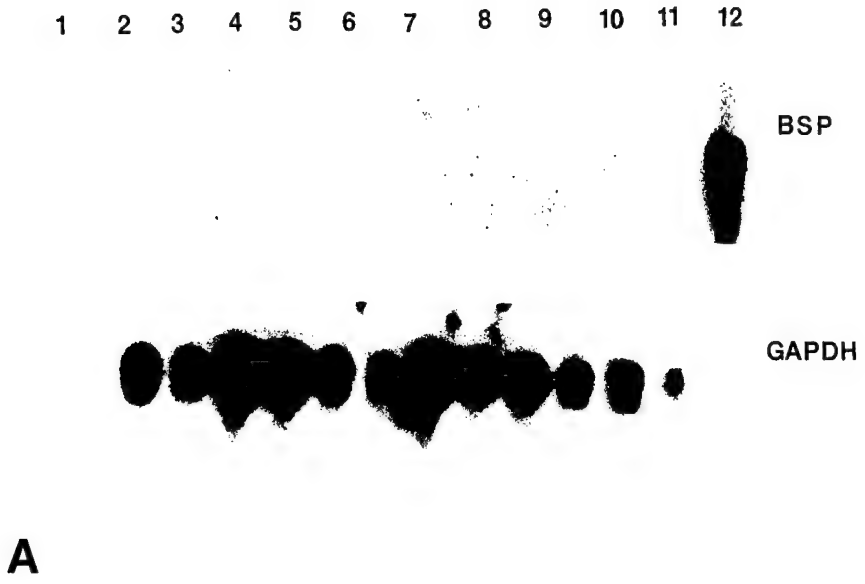


Figure 2

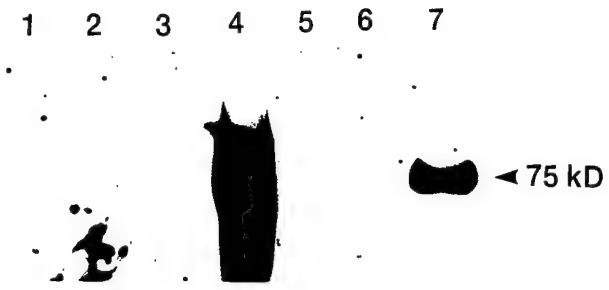




A

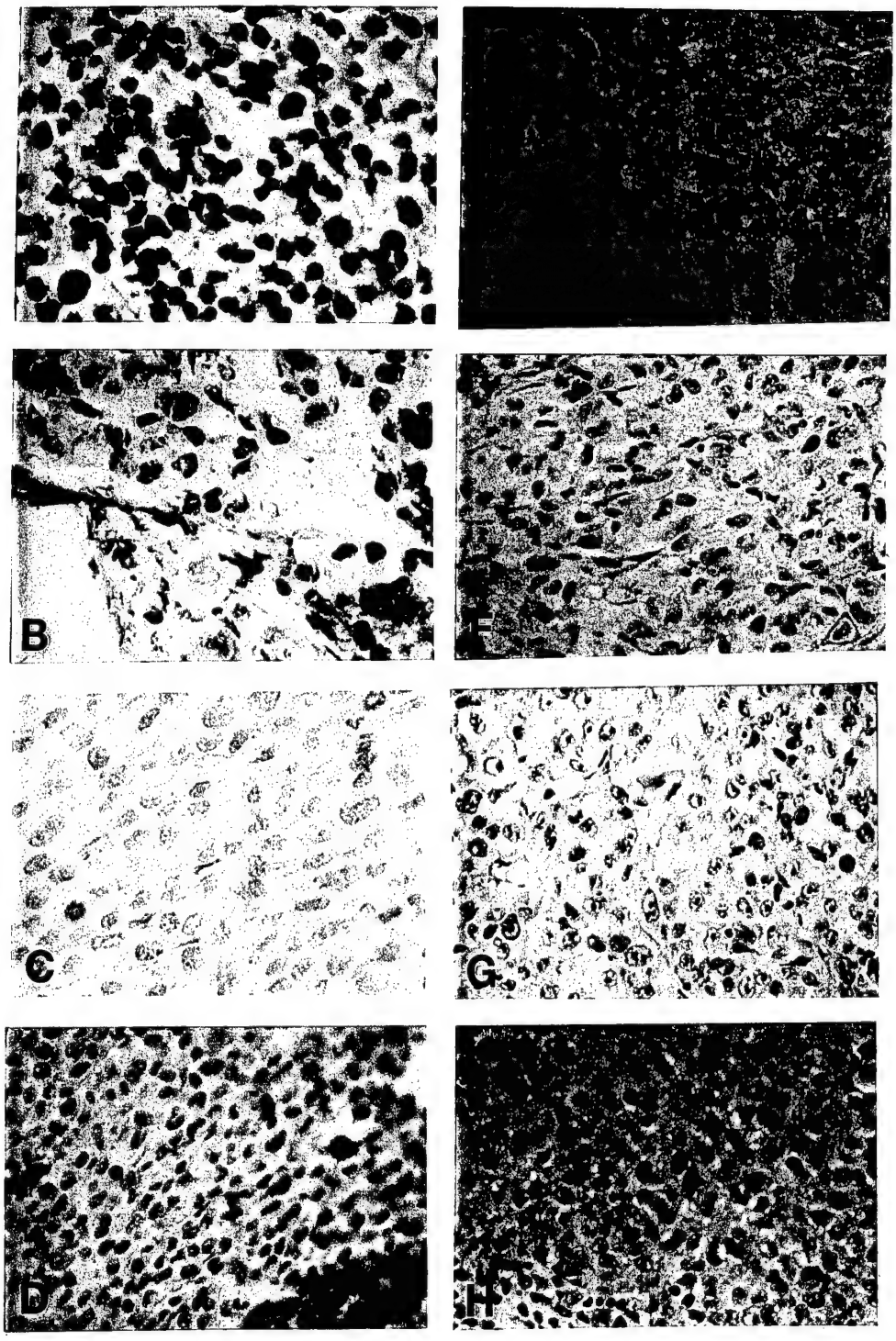
B

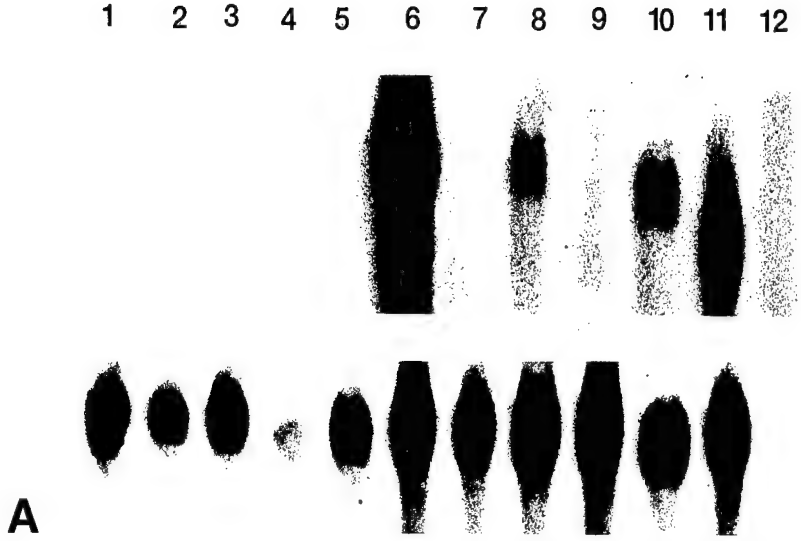
Figure 3C



C

Figure 4

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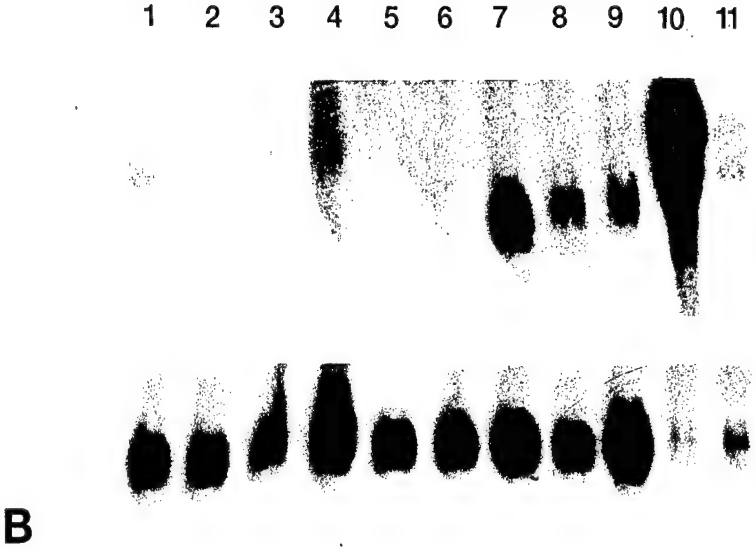
A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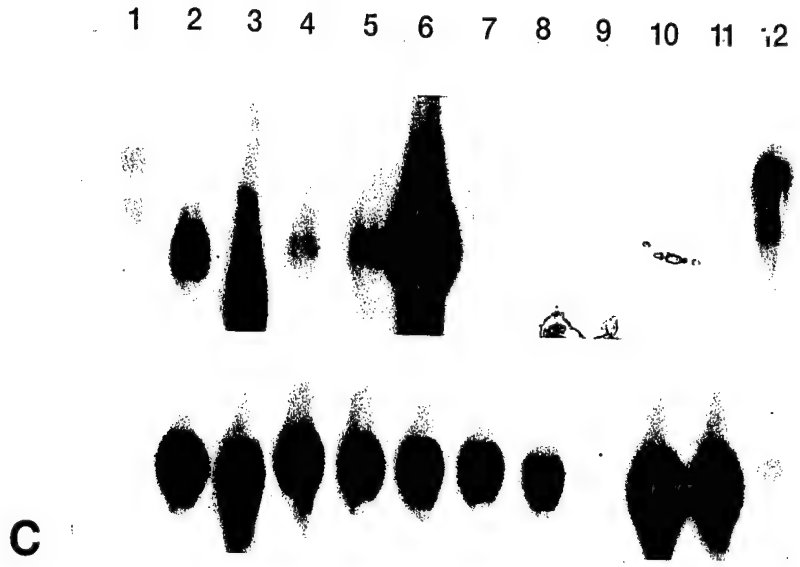
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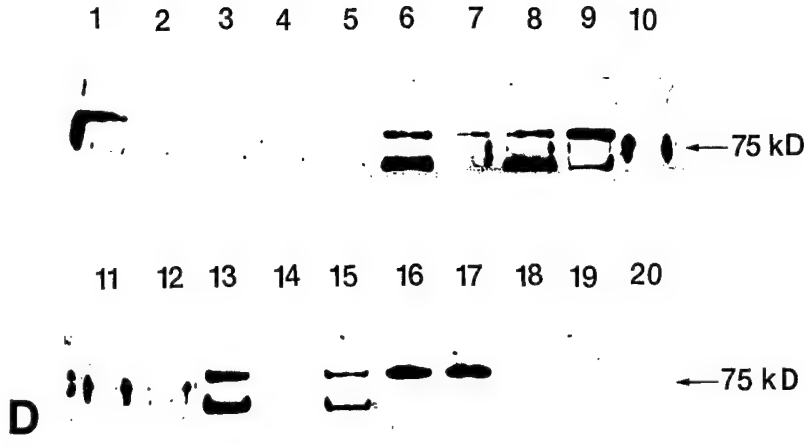
C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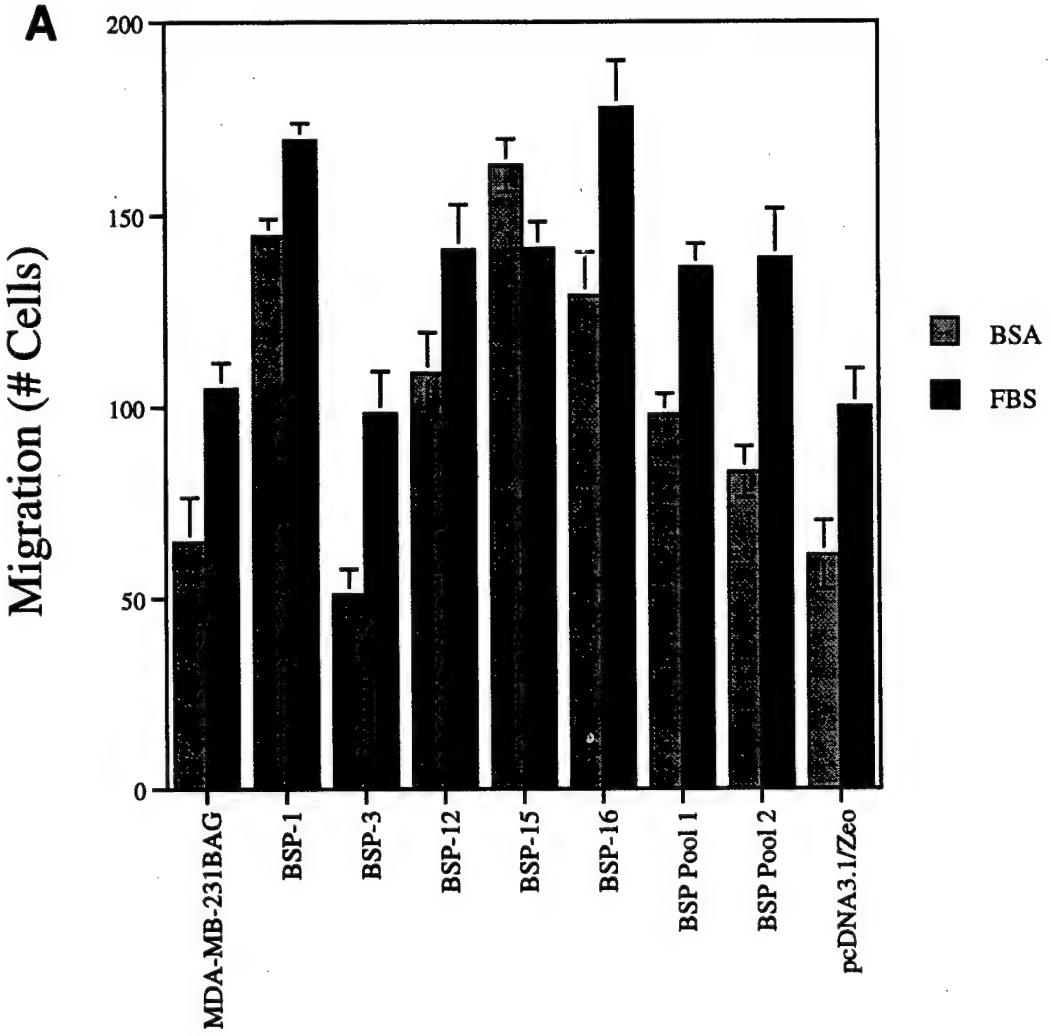
←75 kD
11 12 13 14 15 16 17 18 19 20
←75 kD
D

Figure 6A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Migration (# Cells)
200
150
100
50
0
MDA-MB-231BAG
BSP-1
BSP-3
BSP-12
BSP-15
BSP-16
BSP Pool 1
BSP Pool 2
pcDNA3.1/Zeo
BSA
FBS

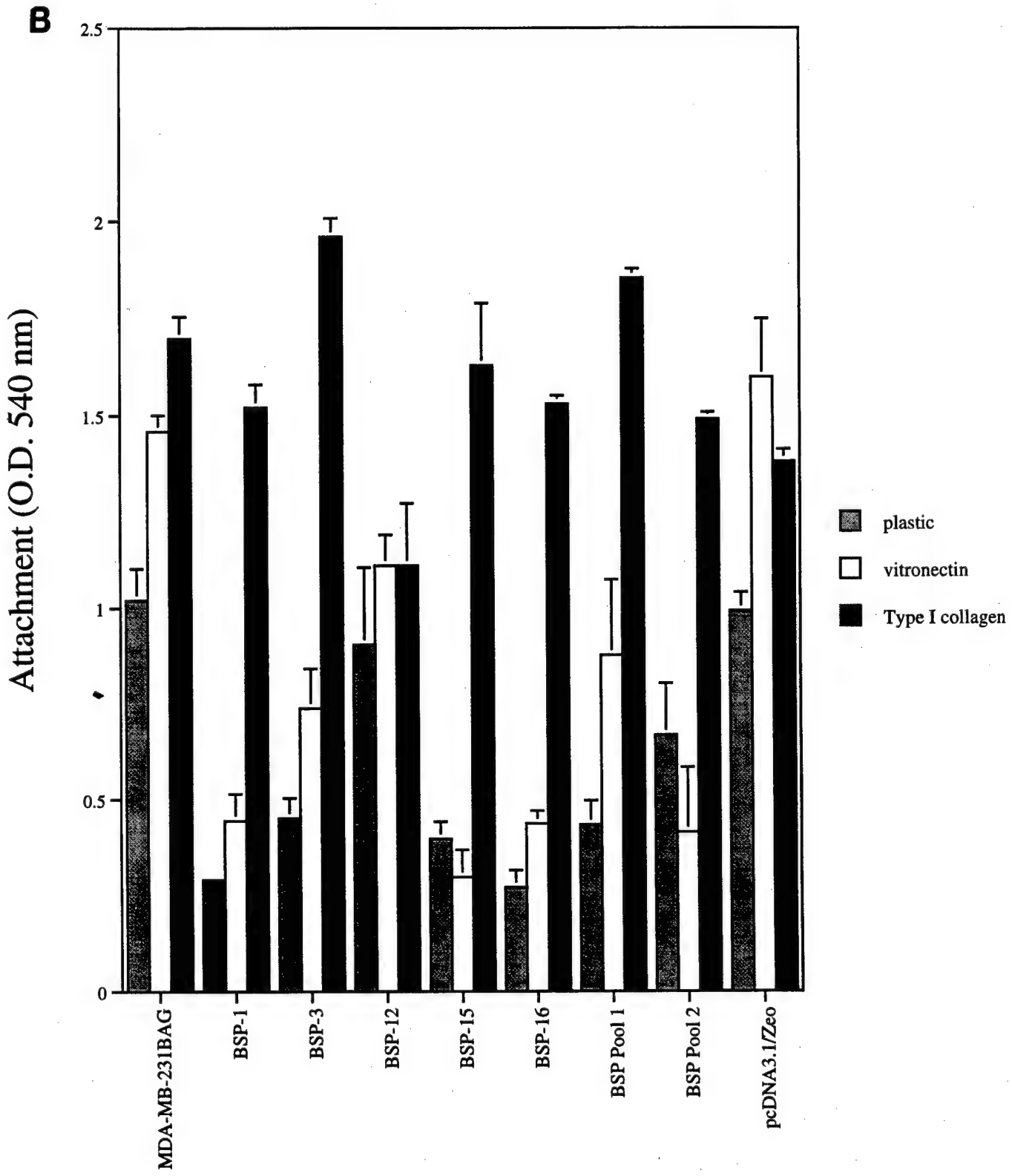
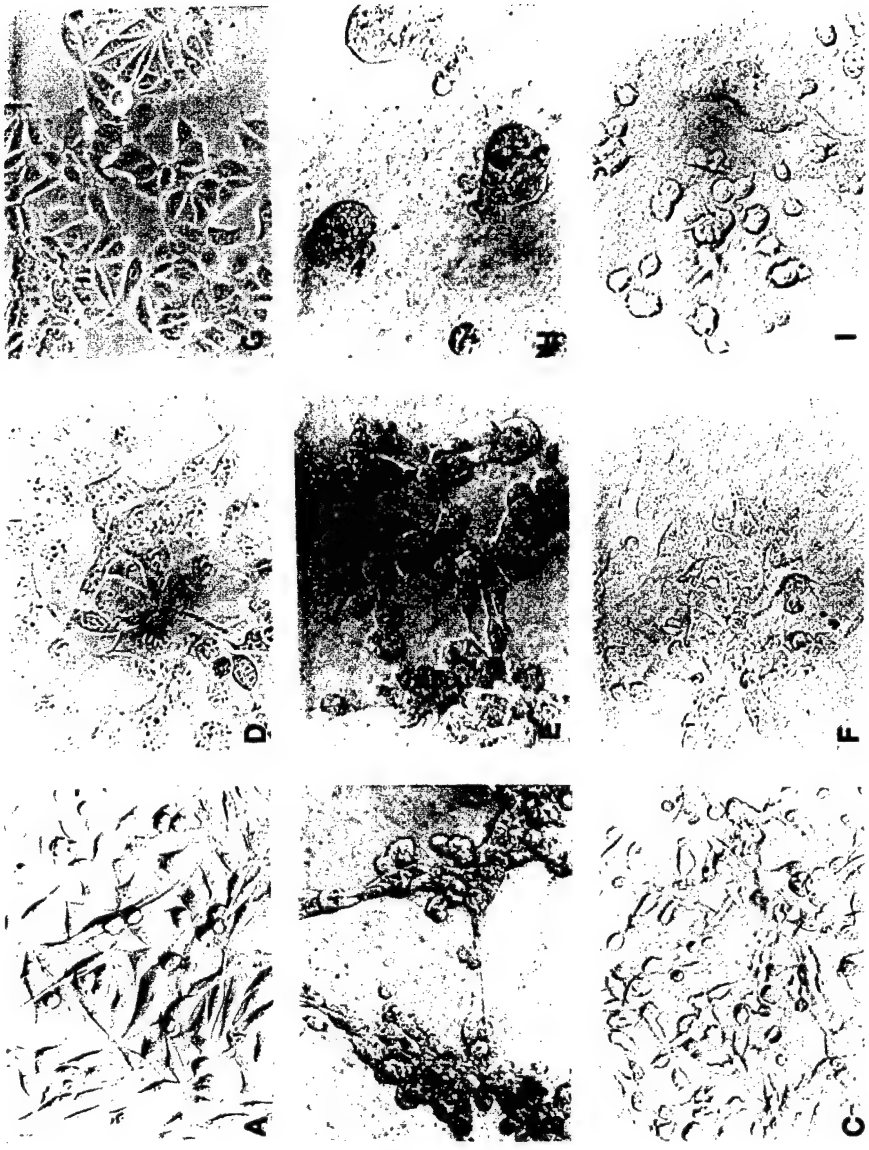
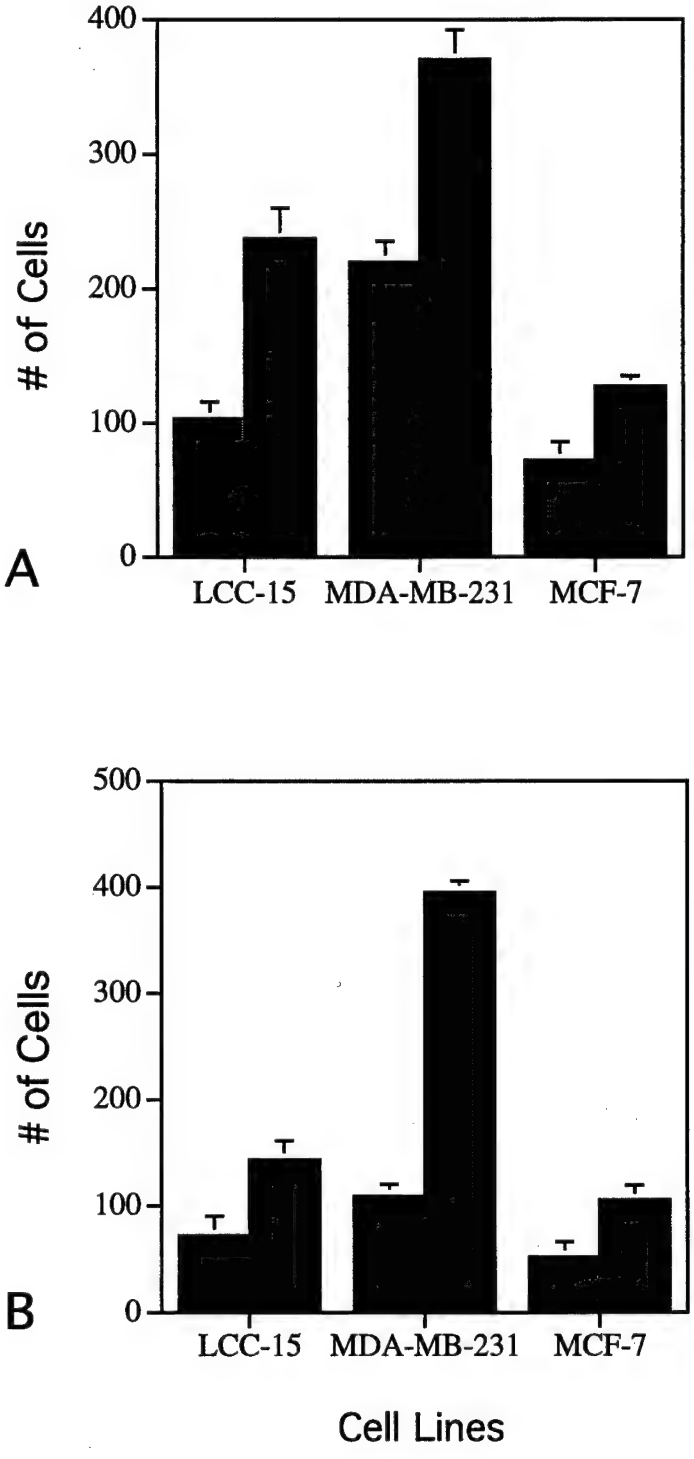

B
Attachment (O.D. 540 nm)
2.5
2
1.5
1
0.5
0
MDA-MB-231BAG
BSP-1
BSP-3
BSP-12
BSP-15
BSP-16
BSP Pool 1
BSP Pool 2
pcDNA3.1/Zeo
plastic
vitronectin
Type I collagen

Figure 7

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300
200
100
0
# of Cells
A
LCC-15
MDA-MB-231
MCF-7
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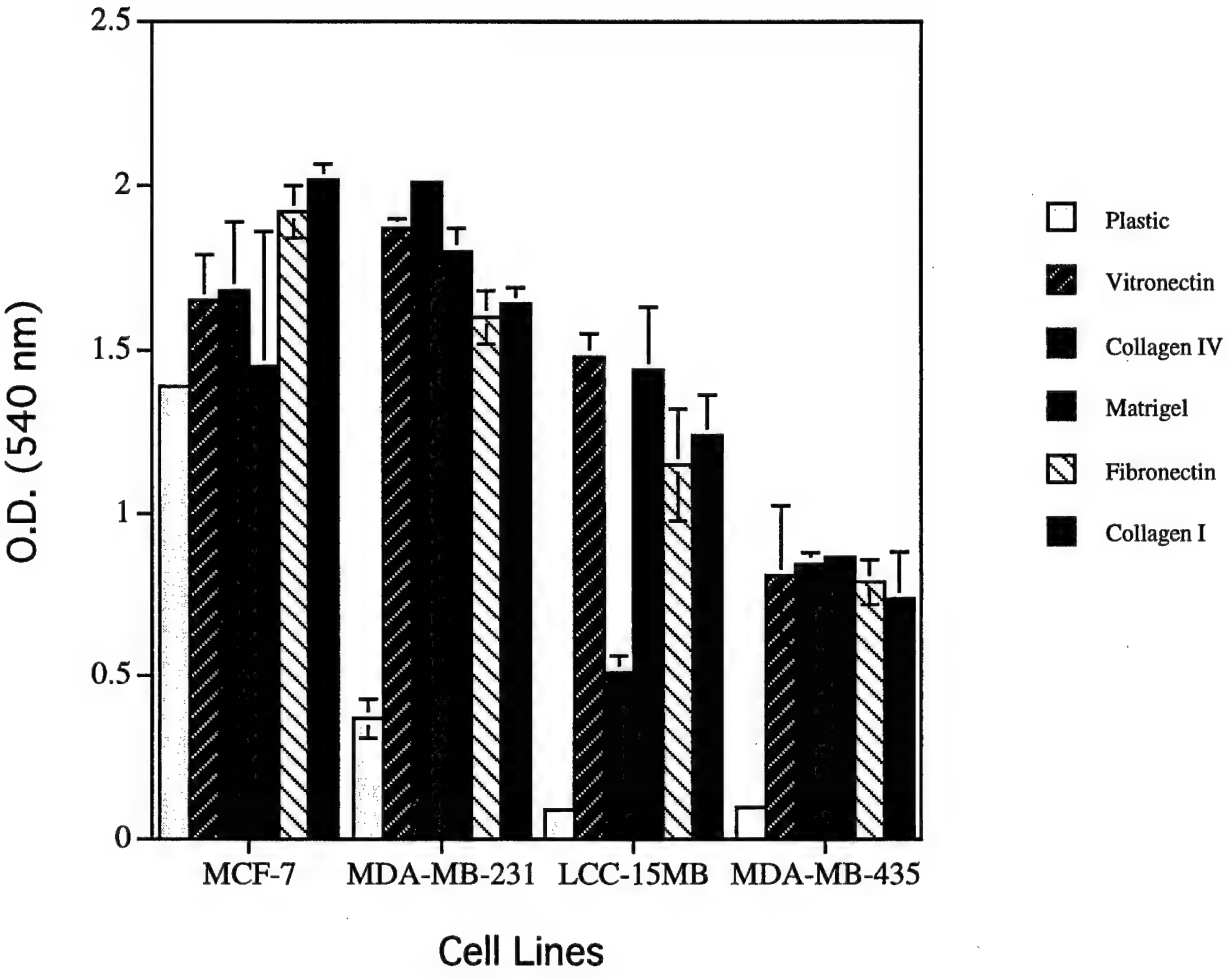
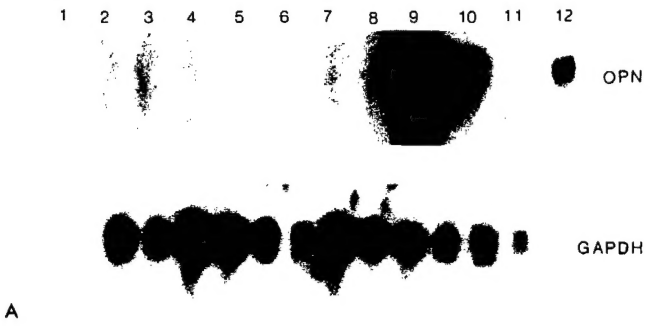
300
200
100
0
# of Cells
B
LCC-15
MDA-MB-231
MCF-7
Cell Lines

Figure 9


2.5
2
1.5
1
0.5
0
O.D. (540 nm)
MCF-7
MDA-MB-231
LCC-15MB
MDA-MB-435
Cell Lines
Plastic
Vitronectin
Collagen IV
Matrigel
Fibronectin
Collagen I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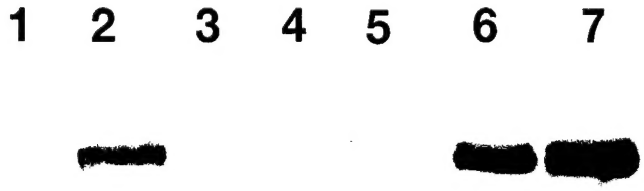
OPN
GAPDH

A

1 2 3 4 5 6 7 8 9 10 11
OPN
GAPDH

B


1 2 3 4 5 6 7

C

Figure 11

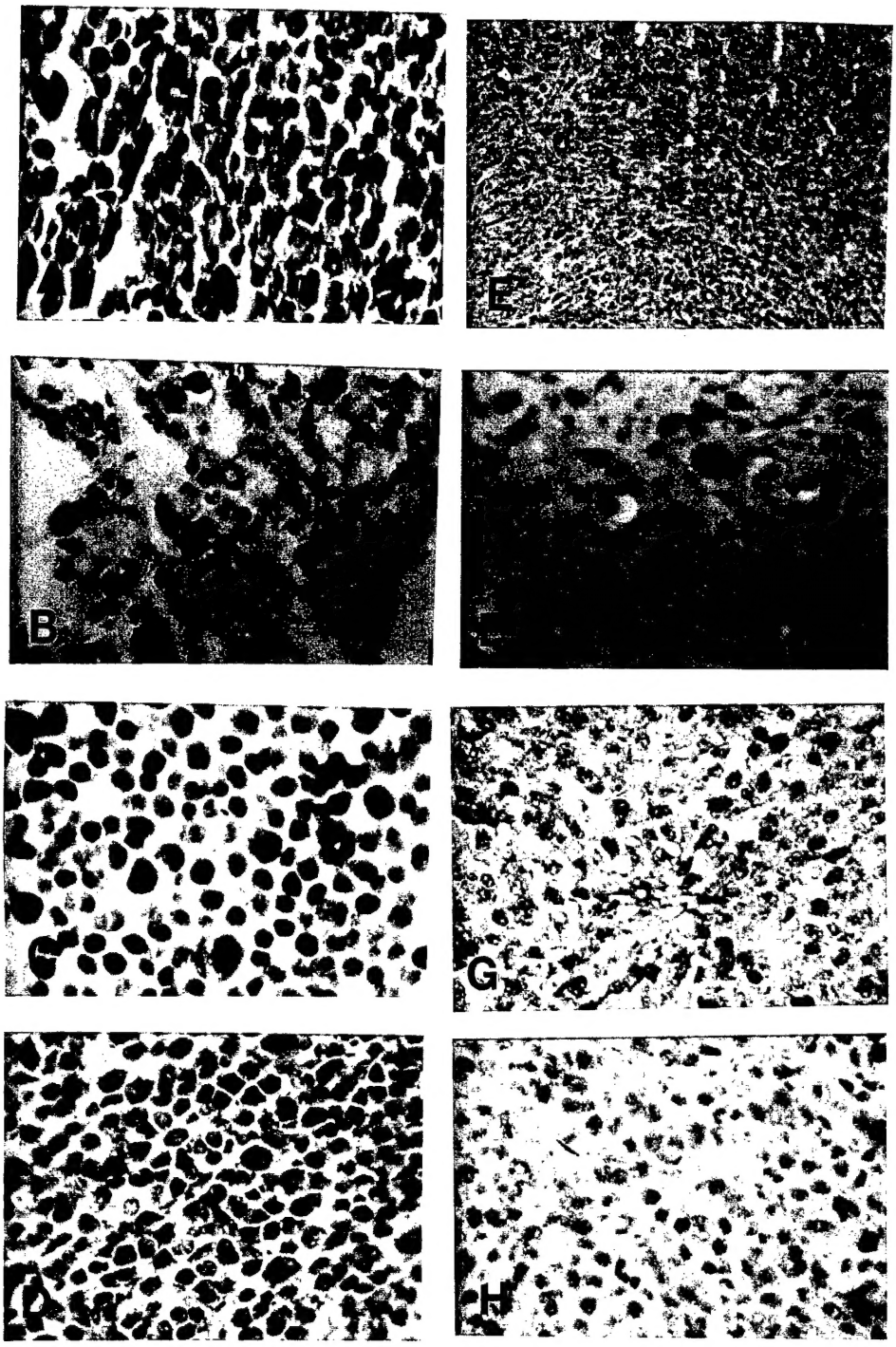


Figure 12

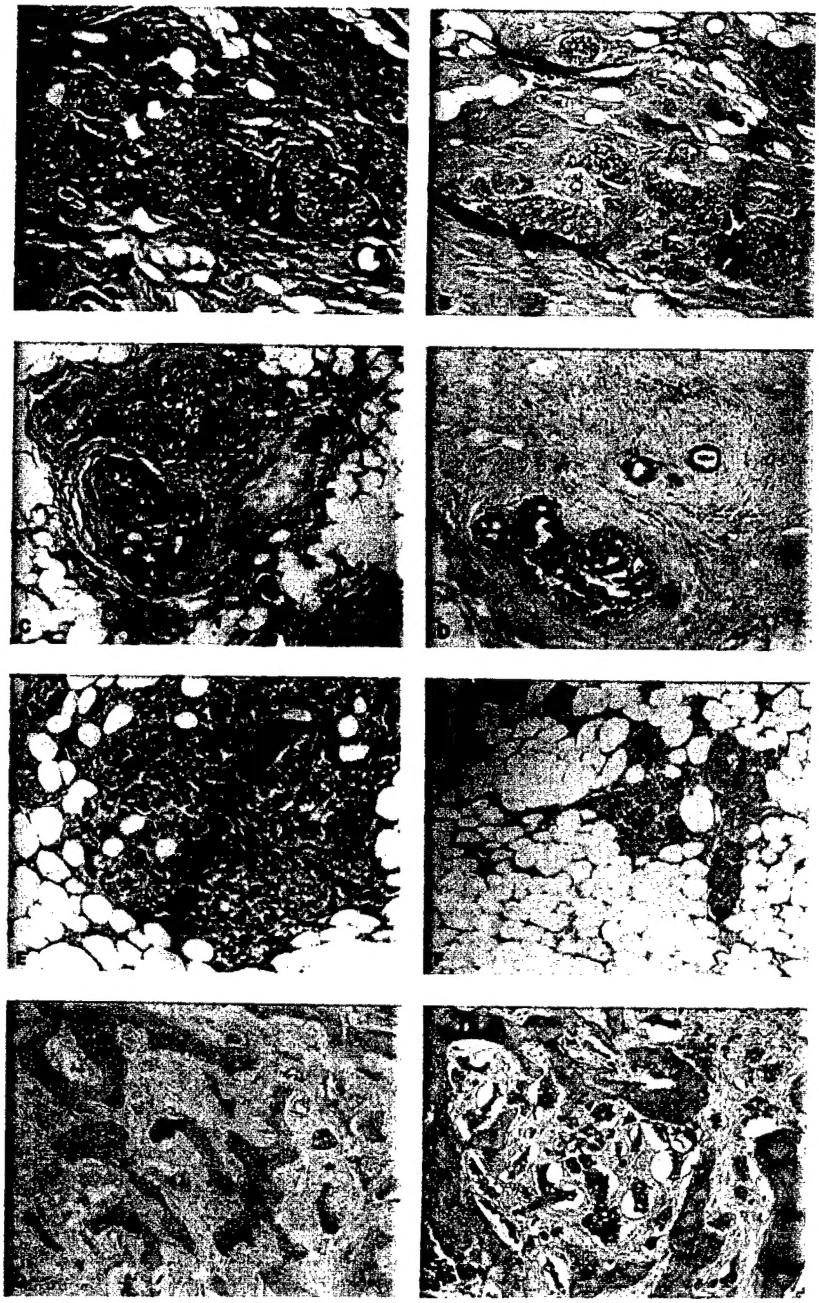
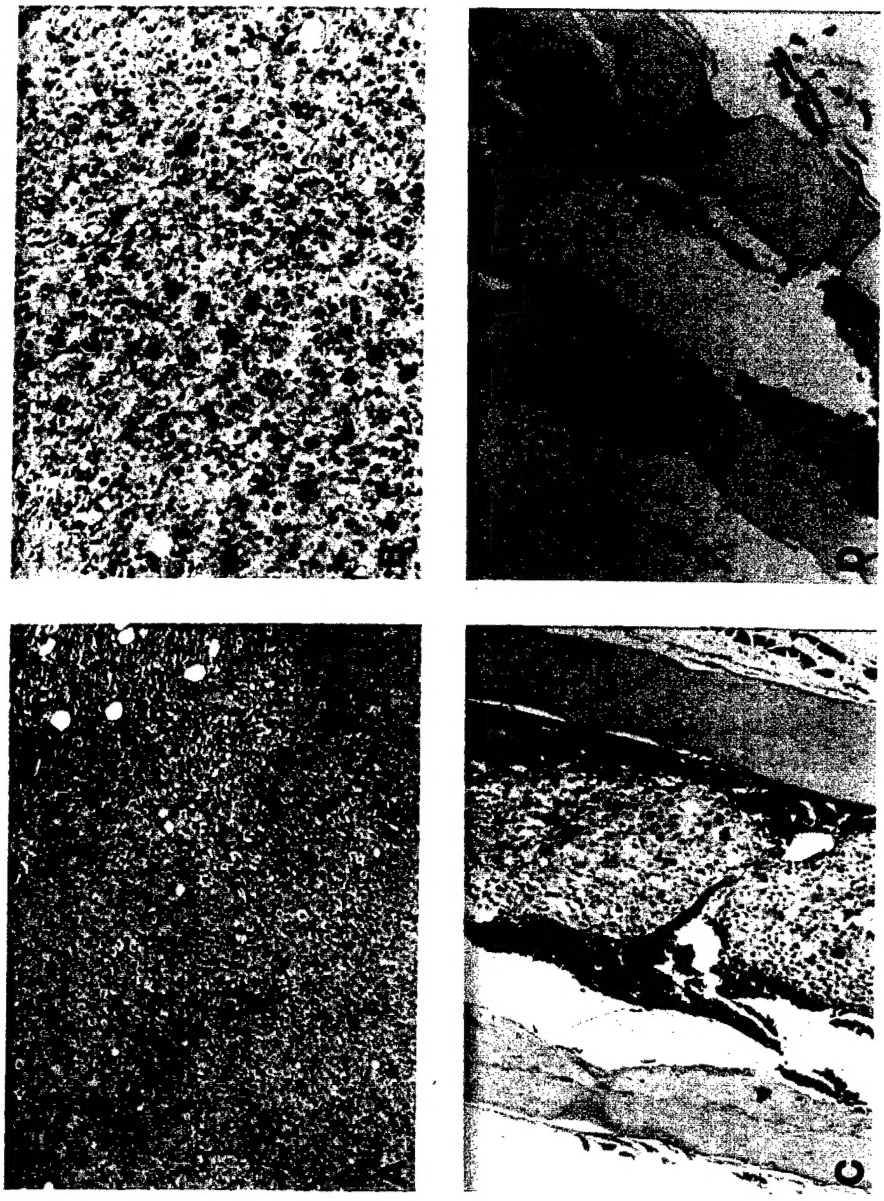



Figure 13